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EXPERIMENTAL ARTICLES

Synthesis of Anabiosis Autoinducers by Non-Spore-Forming Bacteria as a Mechanism Regulating Their Activity in Soil and Subsoil Sedimentary Rocks

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Abstract—Non-spore-forming bacteria of the genera *Arthrobacter* and *Micrococcus*, isolated from permafrost subsoil, were found to produce greater amounts of the d_1 extracellular factor than closely related collection strains isolated from soil. The effect of this factor, responsible for cell transition to anabiosis, was not species-specific. Thus, the d_1 preparation isolated from the culture liquid of the permafrost isolate *Arthrobacter globiformis* 245 produced an effect on the collection strain *Arthrobacter globiformis* B-1112 and also on *Micrococcus luteus* and *Bacillus cereus.* The d_1 preparation from the permafrost isolate of *Arthrobacter* differed from the chemical analogue of this factor, *4-n*-hexylresorcinol, in the level of the induced cell response, which may have resulted from different cell sensitivity to various homologs of alkylhydroxybenzenes contained in the d_1 preparation. Thus, additional evidence was obtained indicating that autoregulation of bacterial growth and development is implemented at the level of intercellular interactions in microbial communities. Abundant production of the d_1 anabiosis-inducing factors by bacteria isolated from permafrost subsoil is probably a result of special antistress mechanisms responsible for the survival of these bacteria under extreme conditions of natural long-term cooling.

Key words: anabiosis, anabiosis autoinducers, bacteria, soil, permafrost subsoil.

Soil microorganisms are known to be able to survive for a long time under environmental conditions unfavorable for active growth, such as a shortage of nutrients, nonoptimal temperature, and other adverse physicochemical factors. Of great interest are microorganisms surviving in frozen subsoil sedimentary rocks for thousands to millions of years and restoring their activity upon thawing [1, 2]. Most of these viable bacteria were found to be the non-spore-forming; they evidently follow a specific strategy of survival under extreme conditions.

One of the ways to survive under these conditions, described mainly for gram-negative bacteria, is the cessation of cell division and a transition to a state characterized by a low level of metabolism. Cells in this state are referred to as *viable* but nonculturable *cells* (VBNC) [3, 4] or *ultramicrobacteria* [5]. Such cells are unable to produce colonies when plated on solid media under standard conditions. After specific resuscitation procedures [4], VNC recovered to growth; however, this was possible only after a definite time of storage. In other words, the reversion of VBNC to active growth is only possible after a relatively short-term nonculturable existence. Another way to survive is cell transition into anabiotic state, in which they can remain viable for a long time. Anabiotic cells are characterized by a low, experimentally undetectable level of metabolic activity and an increased resistance to extreme conditions. Specialized cell forms (spores, conidia, etc.) are well-known examples of anabiotic cells. Recognizing the ecologically important role of nonculturable microorganisms, we however believe that long-term survival of non-spore-forming bacteria in frozen ground is to a great extent due to their ability to produce anabiotic resting forms.

We have shown previously that some non-spore-forming bacteria and yeasts produce cyst-like refractile cells (CRC) displaying the properties of resting forms [6, 7]. The formation of CRC depends on the concentration and activity of specific extracellular metabolites that we termed cell differentiation factors, d_1. They function as autoinducers of anabiosis and belong to alkylhydroxybenzenes (AHB) in some microorganisms [8–10]. It can be assumed that the survival of non-spore-forming bacteria in soil and subsoil sedimentary rocks, including frozen ones, is due to their ability to synthesize similar autoinducers of anabiosis which cause the transition of vegetative cells into the resting

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state. The goal of this study was to assess the level of production of the d_1 factor by non-spore-forming bacteria of the genera *Arthrobacter* and *Micrococcus* isolated from permafrost subsoil sedimentary rocks.

MATERIALS AND METHODS

The isolates of non-spore-forming bacteria of the genera *Arthrobacter* and *Micrococcus* were obtained from 1.5–2 million years old frozen sedimentary rocks sampled in the tundra zone of Kolyma lowland. The sampling procedure, as well as the transportation and storage of the material were performed under sterile conditions [2].

Strains *Arthrobacter globiformis* 245, isolated from permafrost subsoil,[1] and *Arthrobacter globiformis* B-1112 (VKM), isolated from soil, were cultivated at room temperature on trypticase-soybean medium or on synthetic medium composed of (g/l) glucose, 2; K_2HPO_4, 1; KH_2PO_4, 0.1; $(NH_4)_2SO_4$, 0.05; $CaCl_2$, 0.2; and $MgSO_4$, 0.1 (pH 7.0).

The *Micrococcus roseus* collection strain CCM 1145 isolated from soil[2] and an isolate of *Micrococcus* sp. from permafrost subsoil sample were grown on nutrient broth or a deficient medium containing (g/l): glucose, 1; yeast extract, 0.2; $(NH_4)_2SO_4$, 1; KH_2PO_4, 0.1; and trace elements (mg/l): $MgSO_4 \cdot 7H_2O$, 100; $CaCl_2$, 20; $FeCl_3 \cdot 6H_2O$, 0.4; $MnSO_4 \cdot 4H_2O$, 0.4; $ZnSO_4$, 0.4; KI, 0.1; $CuSO_4 \cdot 5H_2O$, 0.04; $Na_2MoO_4 \cdot 2H_2O$, 0.2; NaEDTA, 3 (pH 7.4).

The *Micrococcus luteus* strain NCIMB 13267 was grown on synthetic medium of the following composition (g/l): lithium lactate, 5; NH_4Cl, 4; KH_2PO_4, 4; microelements (mg/l): $MgSO_4 \cdot 7H_2O$, 50; $FeSO_4$, 20; $MnCl_2 \cdot 4H_2O$, 20; $ZnSO_4$, 0.4; $B(OH)_3$, 0.5; $CuSO_4 \cdot 5H_2O$, 0.05; $Na_2MoO_4 \cdot 2H_2O$, 0.2; and growth factors (mg/l): thiamine, 40; methionine, 20 (pH 7.2–7.4).

The *Bacillus cereus* strain 504 (VKM), grown on synthetic medium [7], was also used in some experiments as an additional test-microorganism.

Bacteria were cultivated at 28°C on a shaker (140–160 rpm) in 250-ml flasks containing 50 ml of medium, which was inoculated to an optical density OD = 0.2 with stationary-phase cultures grown on nutrient broth.

Microscopic examinations were performed using a phase-contrast Amplival microscope (Germany). The optical density of microbial suspensions was measured nephelometrically ($\lambda = 660$ nm) on a Specord spectrophotometer in 10-mm cuvettes. The cell dry weight was determined after drying for 24 h at 105°C. Respiratory activity of cell suspensions was measured on a LP7E polarograph (Czechoslovakia) in 1-ml oxygen cell by the Shol'ts–Ostrovskii's method [11].

[1] The strain was provided from the collection of the Department of Soil Science, Moscow State University.

[2] The strains were kindly provided by T.G. Dobrovol'skaya and L.V. Lysak, Department of Soil Science, Moscow State University.

Crude preparations of the d_1 factor were obtained from the culture liquids (bacteria were grown on synthetic medium) by extraction with *n*-butanol added at a ratio of 1 : 1. Butanol was removed on a rotary evaporator, and the residue was extracted using biphasic systems for lipid separation: chloroform–methanol–water (2 : 2 : 1.8 and 2 : 2 : 1 vol/vol). Solvents from both upper and bottom phases were removed in a rotary evaporator, and the residue was dissolved in ethanol. The biological activity of the fractions obtained, introduced as ethanol solutions, was determined (1) qualitatively, from their ability to induce the formation of refractile cells, which was monitored under a phase-contrast microscope in the course of a 1-h exposure, and (2) quantitatively, from their effect on the endogenous respiration of the producer cells (the amount of the factor that inhibited cell respiratory activity by 50% was defined as 1 arbitrarily unit [6]). An equivalent amount of ethanol, which never exceeded 5% (vol/vol), was introduced into control cell suspensions.

The content of AHB in biologically active preparations of the d_1 factor was determined quantitatively using a colorimetric reaction with a diazotized derivative of 3,3'-dimethoxybenzidine (Fast Blue B Salt tetrazotized, FBB, Sigma), which had previously been applied for alkylresorcinol detection in plants and microorganisms [12]. To prepare a solution of the reagent, 5 mg of FBB was dissolved in 10 ml of 5% acetic acid and diluted with five volumes of *n*-propanol. The AHB content was measured directly in the bacterial culture liquid as described previously [10]. The following alkylresorcinols synthesized at the Institute of Organic Chemistry, Russian Academy of Science, were used as references: 5-*n*-pentadecylresorcinol, 4-*n*-decylresorcinol, 4-*n*-hexylresorcinol, 2,5-dibutylresorcinol, and 2-nonyl-5-decylresorcinol.

To assess the inhibiting effect of KCN, cell respiratory activity was determined in a 1-ml cell containing bacterial suspension (0.1 ml) in 0.9 ml of 0.1 M KH_2PO_4 buffer, into which from 5 to 10 μl of a freshly prepared KCN solution of a necessary concentration was added. To determine cytochrome composition, difference absorption spectra (cells reduced with dithionite versus cells oxidized with hydrogen peroxide or ferricyanide) were recorded within a wavelength range from 380 to 650 nm on a Hitachi-557 spectrophotometer (Japan).

The content of d_1 and AHB, as well as changes in cell respiratory activity depending on these factors, were determined in three independent experimental series with each experiment run in triplicate. The results obtained were treated using the STATISTICA software for calculation of standard deviations ($P < 0.05$). The mean arithmetic values are shown in tables and figures; data on standard deviations within the range of 5% are not shown.

To obtain preparations for electron microscopy, cells were precipitated by centrifugation, and, after fix-

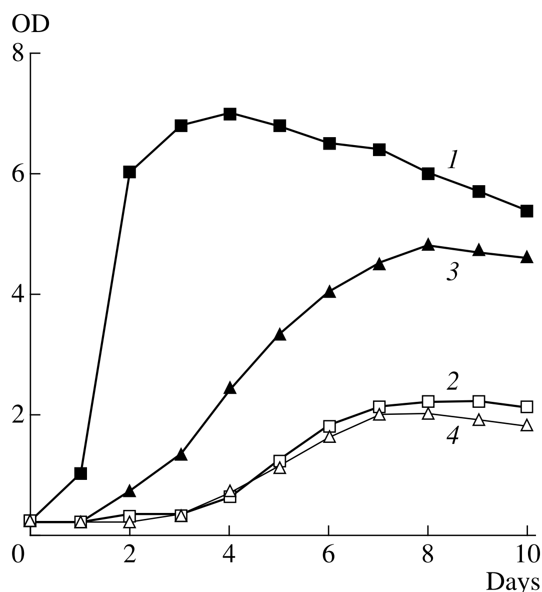

Fig. 1. Growth of *M. roseus* 1145 on (*1*) nutrient broth and (*2*) deficient medium and growth of the *Micrococcus* sp. permafrost isolate on (*3*) nutrient broth and (*4*) deficient medium.

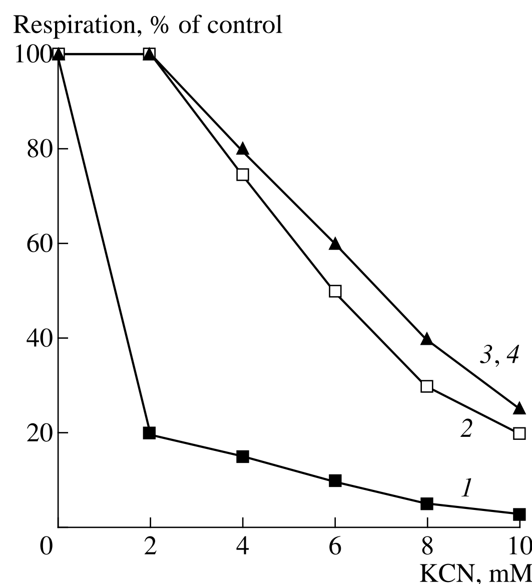

Fig. 2. Inhibition of the respiratory activity of *M. roseus* cells and cells of the *Micrococcus* sp. permafrost isolate by KCN. Designations are the same as in Fig. 1.

ation in a 2.5% solution of glutaraldehyde in 50 μM cacodylate buffer (pH 7.2), they were additionally treated with 0.5% RuO_4 in the same buffer [13]. Thin sections negatively stained with lead citrate were examined in a JEM-1008 electron microscope at a magnification 30 000×.

RESULTS AND DISCUSSION

The *Micrococcus* sp. isolate obtained from permafrost subsoil proved to be closely related to strain *Micrococcus roseus* CCM 1145 [14], according to the colony morphology and physiological and biochemical characteristics. Another strain isolated from permafrost subsoil, *Arthrobacter globiformis* 245, was compared with a related collection strain isolated from soil: *Arthrobacter globiformis* B-1112.

Comparison of the collection strain *Arthrobacter globiformis* B-1112 and *Arthrobacter globiformis* 245 isolated from permafrost subsoil revealed no apparent distinctions between them: the maximum specific growth rates were 0.25 and 0.27 h_{-1}, and the biomass yields were 1.2 and 1.4 g/l, respectively. In contrast, *M. roseus* 1145 and the *Micrococcus* sp. from permafrost subsoil differed significantly. On rich medium, nutrient broth, the collection strain of *M. roseus* displayed a short lag phase (4 h), a high maximum specific growth rate (μ_{max} = 0.39 h^{-1}), and a high biomass yield (3.2 g/l). The *Micrococcus* sp. isolate obtained from permafrost subsoil displayed a prolonged lag phase (24 h), a low μ_{max} = 0.11 h^{-1}, and a 1.7-fold lower biomass yield. On medium deficient for the major sources of nutrition, both the collection strain of *M. roseus* and the isolate were characterized by a substantial decrease in the growth rate (μ_{max} = 0.005 h^{-1}) and a low biomass yield, 0.9 g/l (Fig. 1).

In response to starvation, which may be considered a stress situation for microorganisms, not only does their growth rate decrease, but they also develop a defence reaction, gaining resistance to unfavorable conditions. To assess cell resistance, their reaction to the effect of respiratory poison, cyanide, was studied. In *M. roseus* 1145, the respiratory chain enzymes exhibited the lowest resistance to cyanide (1 mM) during exponential and linear growth of the culture on nutrient broth (Fig. 2). The resistance of stationary-phase cells increased; at a cyanide concentration of 1 mM, the cell respiratory activity was 20 to 30% reduced. In contrast, the cells of the *Micrococcus* sp. permafrost isolate growing exponentially on nutrient broth were highly resistant to the effect of the inhibitor: their respiratory activity was 50% inhibited only at a KCN concentration of about 6 mM (Fig. 2). On a deficient medium, both micrococci showed slow growth and were resistant to cyanide. In cyanide-resistant micrococci, either grown on a deficient medium or exhibiting a constitutively low growth rate, the cytochrome $a_{(601)}$ absorption maxima were extremely low, as distinct from cyanide-sensitive micrococci. Such a correlation between the growth rate, dependent on the cultivations conditions, and the resistance to KCN was previously described for *Rhodococcus minimus* [15]. The constitutively low growth rate of the permafrost isolate, which was independent of the medium composition (nutrient broth or deficient medium) and correlated with increased resistance to stresses, is probably one of the factors that promote the survival of these and may be other bacteria under extreme conditions.

Table 1. Content of the extracellular d_1 factor and AHB* per unit of volume and biomass in the isolate *A. globiformis* 245 from permafrost subsoil and in the collection strain *A. globiformis* B-1112

Culture	Content of extracellular d_1 factor, act. units/l	Yield of extracellular d_1 factor, act. units/g cells	Content of AHB in preparations of extracellular d_1 factor, mg/ml**
A. globiformis B-1112	8	3.6	0.07
A. globiformis 245	100	38.5	1

* In crude preparations isolated from equal volumes of the culture liquid.
** One milliliter of the preparation was obtained from 5 l of the culture liquid.

Table 2. Content of extracellular d_1 factor and AHB* per unit of volume and biomass in the *Micrococcus* sp. isolate from permafrost subsoil and in the collection strain *M. roseus* CCM 1145

Culture	Content of extracellular d_1^* factor, act. units/l	Yield of extracellular d_1^*, factor, act. units/g cells	Content of extracellular AHB, mg/l	Yield of extracellular AHB, mg/g dry cells
M. roseus CCM 1145	13.2	14.6	1.2	1.3
Micrococcus sp	22.4	24.8	2	2.2

* In crude preparations isolated from equal volumes of the culture liquid.

Table 3. Sensitivity of cells* of the collection strains of *A. globiformis*, *M. luteus*, and *B. cereus* to the extracellular d_1 factor from *Arthrobacter globiformis* 245 (the isolate from permafrost subsoil) and to d_1 analogue, 4-hexylresorcinol

Culture	Amount sufficient for 50% inhibition of cell respiration by		Concentration of 4-hexylresorcinol inducing formation of anabiotic cells*, M
	d_1 factor of *Arthrobacter globiformis* 245, mg AHB/ml	analogue of d_1 factor, 4-hexylresorcinol, M	
A. globiformis B-1112	1.2–1.3	2.5×10^{-4}–3.0×10^{-4}	8.0×10^{-4}–10×10^{-4}
M. luteus	2.0	7.5×10^{-3}–8.0×10^{-5}	2.5×10^{-4}–3.0×10^{-4}
B. cereus	1.4	2.0×10^{-4}–3.0×10^{-4}	10.3×10^{-4}–25×10^{-4}

* Cells from the phase of linear growth (medium pH 7.2).

Table 4. Sensitivity of cells* of *M. luteus* and *A. globiformis* B-1112 to various chemical analogues of the d_1 factor

Analogue	Concentration sufficient for	
	50% inhibition of *M. luteus* cell respiration, M	formation of *A. globiformis* B-1112 anabiotic cells, M
4-*n*-Hexylresorcinol	8.0×10^{-5}	10×10^{-4}
4-*n*-Decylresorcinol	40.0×10^{-5}	5.0×10^{-4}
5-*n*-Decylresorcinol	5.1×10^{-5}	5.0×10^{-4}
2,5-Dibutylresorcinol	13.0×10^{-5}	10×10^{-4}
2-Nonyl-5-decylresorcinol	28.2×10^{-5}	2.0×10^{-4}

* Cells from the phase of linear growth (medium pH 7.2).

It is well-known that the anabiotic forms of microorganisms, such as endospores, conidia, etc., are most resistant to various adverse impacts. In non-spore-forming microorganisms, the formation of anabiotic cells is induced at an increased concentration of extracellular metabolites, autoinducers of anabiosis; this was demonstrated for a number of bacteria and yeasts [6, 7]. In further experiments, we tried to isolate these autoregulatory factors and compare the level of their production by arthrobacters and micrococci from permafrost subsoil with the production level characteristic of the collection strains.

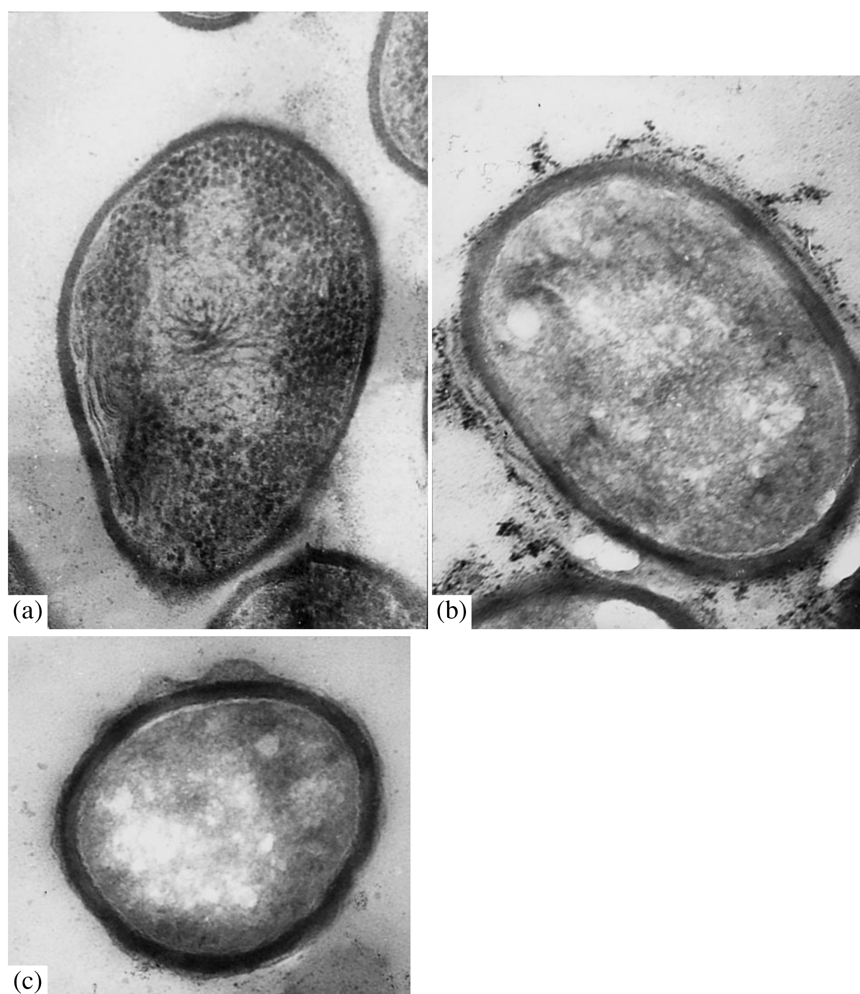

Fig. 3. Thin sections of cells of the *A. globiformis* 245 permafrost isolate: (a) a vegetative cell of *A. globiformis* 245 (3-day culture grown on trypticase-soybean medium; 54000×); (b) and (c), anabiotic cells of *A. globiformis* 245 whose formation was induced (b) by a chemical AHB analogue, 4-*n*-hexylresorcinol or (c) by a crude preparation of the d_1 factor, autoinducer of anabiosis (54000×).

The d_1 extracellular factors (autoinducers of anabiosis) were isolated from 5-l portions of the culture liquids of stationary-phase cultures of *A. globiformis* 245 (the permafrost isolate) and *A. globiformis* B-1112 (a collection strain) as described in [8–10] and dissolved in 1 ml of ethanol. The biological activity of the d_1 factor (the effect on the producer respiration), expressed in arbitrarily units per 1 l of the culture liquid or 1 g of dry cells, was an order of magnitude higher in the *A. globiformis* permafrost isolate than in the *A. globiformis* collection strain (Table 1). In some of the previously studied microorganisms, alkylhydroxybenzenes (AHB) were found to be the active substance of the d_1 factors; however, they varied in their structure in different producers [8–10]. The colorimetric reaction with FBB is specific for AHB (alkylresorcinols) [12], and they were detected by this method in biologically active preparations of the d_1 factors obtained from the bacteria studied. The specific concentration of AHB in the d_1 preparation from the *A. globiformis* permafrost isolate was an order of magnitude higher than in the d_1 preparation from the collection *A. globiformis* strain. This correlated with the levels of autoregulatory substances measured in units of biological activity.

The d_1 autoregulatory factors were also isolated from the cultures of the permafrost isolate of *M. roseus* and the matching collection strain grown on a deficient medium. The biological activity of the preparations obtained was studied (Table 2) and their AHB content was determined using the colorimetric quantitative reaction with FBB [10, 12] and taking into account the previous evidence concerning the alkylhydroxybenzene nature and the d_1 factor in *Micrococcus luteus* and data on the dynamics of its synthesis [10]. In the isolate of micrococci from permafrost subsoil, both the content of the d_1 factor (as judged from the biological activity) and AHB (as judged from the reaction with FBB) were higher than in the collection strain (Table 2). The formation of the cyst-like, refractile cells was observed after the addition of the d_1 factor from *A. globiformis*

245 (the isolate from permafrost subsoil) to the producer culture, as well as after the addition of some chemical analogues of AHB.

Electron microscopy of the *A. globiformis* 245 anabiotic cells obtained under the influence of a chemical AHB analogue, 4-*n*-hexylresorcinol (Fig. 3b), or under the influence of the d_1 factor from the culture liquid (Fig. 3c) showed that the electron density of their cytoplasm decreased, the nucleoid region was poorly expressed, and the cell walls thickened as compared to the vegetative cells. Anabiotic cells with similar features were previously found in *A. globiformis* suspensions after autolysis [16]. Cyst-like refractile cells of other bacteria [7], whose formation was induced by the d_1 factors, displayed the same features.

Thus, the *A. globiformis* and *Micrococcus* sp. isolates from permafrost subsoil produced higher amount of autoinducers of anabiosis than the related collection strains of these bacteria.

The effect of these compounds was not species-specific, which is ecologically important. For example, the d_1 preparation from *A. globiformis* 245 had an effect both on the collection strain *A. globiformis* B-1112 and on other microorganisms, *M. luteus* and *B. cereus.* The same was characteristic of the d_1 chemical analogue, 4-*n*-hexylresorcinol (Table 3), although some quantitative distinctions were revealed. Vegetative *M. luteus* cells at the stage of linear growth were more sensitive to the chemical analogue of the d_1 factor than the *A. globiformis* and *B. cereus* collection strains; i.e., lower concentrations of the analogue inhibited endogenous respiration and induced cell transition into anabiotic state (Table 3). In micrococci, the formation of resting refractile cells was observed at a low concentrations of the analogue (Table 3), which can probably be explained by a high intracellular level of the d_1 factor in these bacteria (2.3 mg/g dry cell weight [10]). In pseudomonads, for example, the level of AHB in cells is an order of magnitude lower [12, 17]. Because of this, in micrococci, the threshold level of the autoregulatory substances that is required for cell transition to anabiosis is achieved after the addition of a lesser amount of exogenous AHB than in other microorganisms. Hence, in bacteria of different genera, the level of sensitivity to these autoregulatory substances may depend on the intracellular concentrations of the autoinducers of anabiosis.

It should be noted that *M. luteus* cells proved to be less sensitive to the d_1 preparations isolated from the permafrost isolate of *A. globiformis* (Table 3) than to the chemical analogue studied. In different microorganisms, the AHB isomers and homologs constituting the d_1 factor differ in their structure [8–10]; this probably accounts for different sensitivity to them. This was confirmed in experiments with *M. luteus* and *A. globiformis* B-1112. Inhibition of respiratory activity in the *M. luteus* cells or transition of the *A. globiformis* cells to anabiosis were induced by different concentrations of AHB varying in the position of the substituting group in their ring or in the length of their alkyl radicals (Table 4). The effect of the compounds studied on micrococci and arthrobacters probably depends on the peculiarities of the membrane structure of recipient microorganisms.

Thus, the isolates of *Micrococcus* sp. and of *Arthrobacter globiformis* obtained from permafrost subsoil differed in certain physiological properties from the collection strains of the same genera. For example, the *Micrococcus* isolate displayed a constitutively low growth rate when cultivated on both enriched and deficient media, which correlated with its increased resistance to adverse impacts (cyanide in our experiments). It may well be that slow growth, which is genetically determined or induced by external factors, is related to the resistance to various stress conditions; analysis of the mechanisms underlying this phenomenon may be the subject of further studies. The formation of the microbial community under conditions of long-term effect of low temperatures is probably accompanied by a selection of clones (variants) more resistant to the adverse conditions; these clones may be capable of long-term survival in the frozen ground due to a more pronounced capacity for autoinducer-promoted transition to anabiosis.

Of interest is the fact that in the isolates of *Arthrobacter globiformis* and *Micrococcus* sp. obtained from permafrost subsoil, the production of the extracellular d_1 factor responsible for cell transition to anabiotic state was 10- and 20-fold higher than in the collection strains. In the isolate of *Micrococcus* sp., in particular, the high level of the autoregulatory substances may account for its constitutively slow growth, as well as for its resistance and long-term survival in the frozen ground.

The d_1 factors with anabiosis-inducing activity were also revealed in *A. globiformis* 245 and *A. globiformis* B-1112, which suggests that universal mechanisms are responsible for the autoregulation of bacterial growth and development under adverse conditions. In various microorganisms, the d_1 factors are composed of alkylhydroxybenzenes differing in their structure [8–10]; nevertheless, their activity is not species-specific; i.e., the d_1 factors have an effect not only on the producer cells, but also on cells of other microorganisms. However, various microorganisms exhibit different sensitivity to autoregulatory compounds; these differences are related both to the structure of these compounds and to the recipient properties. In general, the results obtained in this work support the suggestion that under a long-term ecological impact, antistress mechanisms develop in bacterial cells, and clones with higher resistance remain viable as anabiotic resting forms. In the microbial communities of the many-years-long frozen subsoil sedimentary rocks, such clones are actively selected for, and they can restore metabolic activity more readily than the members of the tundra soil microbial communities under conditions of artificial or natural sporadic freeze-thawing [2].

The results of this study provide an insight into the functioning of the microbial community consisting of both producers and recipients of extracellular autoinducers of anabiosis. Ya. Khudyakov was the first to hypothesize that autoregulation in the soil microbial communities is a result of chemical intercellular interactions [18]. He suggested that fluctuations in the microbial population density are caused by periodical changes in the concentration of a complex of phenol compounds, *periodin*, capable of inhibiting all soil microorganisms. We suggest that the extracellular d_1 factors may be considered one of the periodin components which perform regulatory functions at the level of intercellular interactions in the microbial communities and ecosystems.

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